METHOD FOR OBTAINING CHROMOSOMES

James P. Bogart

Department of Zoology, The University of Texas.

It is very easy to obtain chromosomes from anuran amphibians. Amphibians have very large chromosomes which can easily be seen with an ordinary microscope. The method used has been tested in the laboratory and also at collecting sites. All that is required are a few chemicals and simple equipment.

Some solutions have to be prepared in advance but these solutions can be kept for several months. The stain is produced by combining 3 grams of ynthetic orcein with 100 cc. of warm 70% acetic acid and refluxed gently for about one hour. The resulting stain is filtered to remove any undissolved stain particles. This process produces a saturated stain solution. To produce the colchicine solution, 50 cc. of distilled water are placed in a clean pyrex container which is then autoclaved and cooled. 12.5 mg. of colchicine are added to the container to produce a stock solution of colchicine. The solution should be kept light tight by wrapping the container in aluminum foil and it should be refrigerated. If refrigeration is not available, the colchicine may be added to sterile distilled water a short time before it is to be used. The solution will last for several days if sterile technique is employed. Stock solutions of 100% acetic acid, distilled water, and absolute alcohol are required.

A glass spot-plate is used to contain the solutions. It must be very clean and dust free. Lens paper is used to clean the spot-plate first with alcohol and then with distilled water. Six drops of distilled water are then placed in each depression of the spot-plate. A sterile pipette is used to draw up some of the stock solution of colchicine. Two drops of the stock solution are added to each depression to produce a final concentration of .008%

colchicine. This solution may be used for obtaining chromosomes from both corneal epithelium or tadpole tail tips.

To obtain chromosomes from corneal epithelium, a frog is selected and his eyes should be washed with distilled water to ensure that there are no dirt particles around the eyes. The frog is then pithed and the eyes are dissected using razor blade fragments mounted in glass tubes with sealing wax. Care must be taken not to rupture the eye. The eye is then transferred to one depression in the spot-plate and placed, cornea down, in the solution for two hours. When two hours have elapsed, the eye is suspended over glacial acetic acid for one minute and then placed in distilled water for about one minute. The cornea becomes cloudy. The eye is then placed under a dissecting microscope and the cornea is removed by scraping the eye with a scalpel. The cornea is then placed on a clean slide with a drop of stain. The cover slip is added and any air bubbles are gently forced out using the end of a match stick. This also helps to spread the cornea. A folded piece of bibulous paper is used to hold the slide in place and the cover slip is held secure. The squash is accomplished by exerting a great deal of pressure on the cover slip with the thumb. The cover slip is sealed with cover glass cement.

If tadpole tail tips are used, the procedure is the same but it is not necessary to fix the tadpole tail by suspending it over acetic acid. The tail is transferred from the colchicine solution directly to the slide with a drop of stain. The same squash pretreatment time of two hours applies for tadpole tail tips as well as for eyes.

The slides are scanned and the best figures are photographed. In some cells the chromosomes are spread as they are shown in Figure 1. This is a metaphase C configuration which is an exploded metaphase induced by colchicine. There is no metaphase plate if colchicine is used. The squash in Figure 1 is from a corneal cell of *Bufo retiformis* from Arizona in the United States. There are 22 chromosomes in this species. Most of the chromosomes are metacentric and the secondary constrictions are very evident in this squash. Secondary constrictions have proven to be very useful in the determination of relationships in the genus *Bufo*.

A typical squash from a tadpole tail tip is presented in Figure 2. The tadpole in this case is a hybrid. The female of the combination was *Bufo americanus* from Minnesota in the United States and the male was *Bufo luetkeni* from Mexico. Chromosomes in hybrids provide a good method to compare the chromosomes between the two parental species. In a hybrid cell, the chromosomes of the two parents are subjected to exactly the same treatment and they are at the same stage of contraction. True hybrids must have an equal contribution of chromosomes from both species. If the two species did not contribute only their haploid number of chromosomes, then

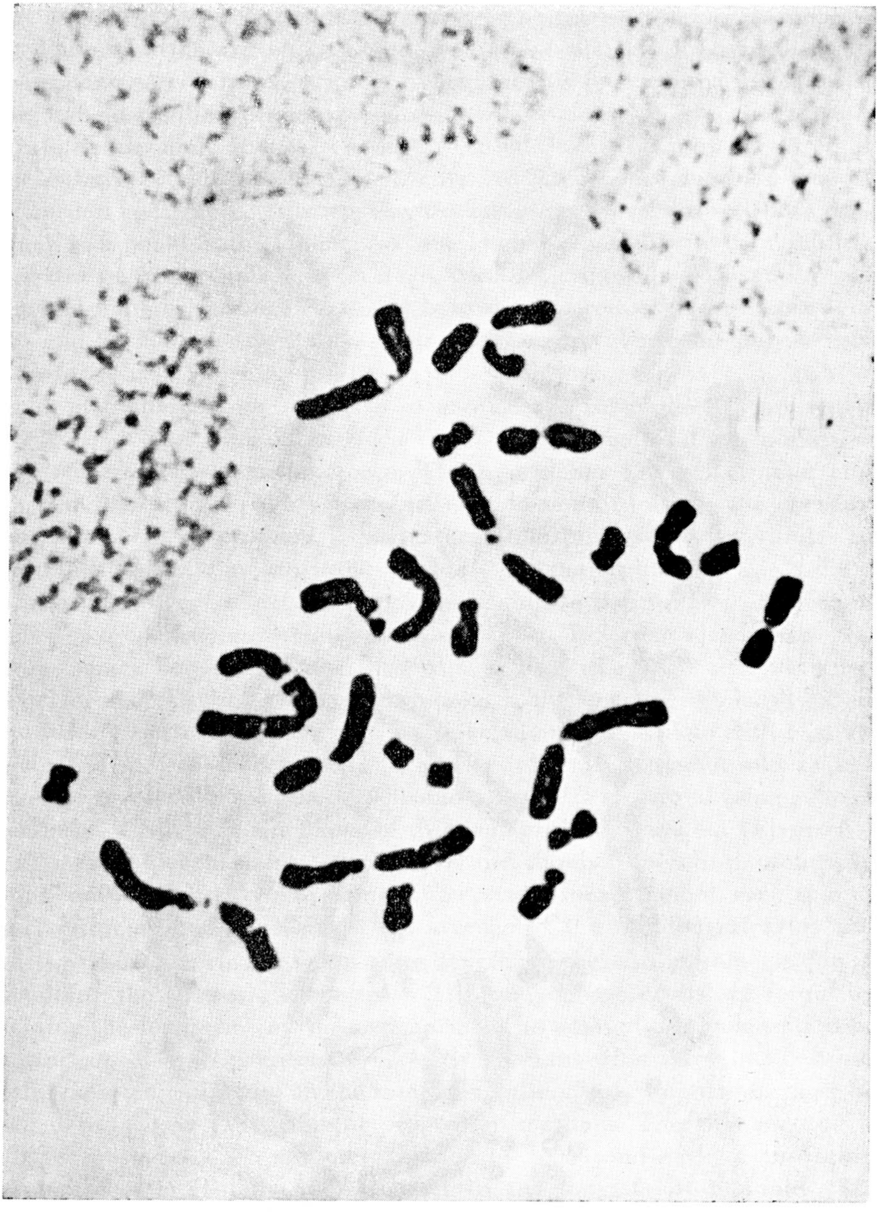

FIGURE 1. *Bufo retiformis.*

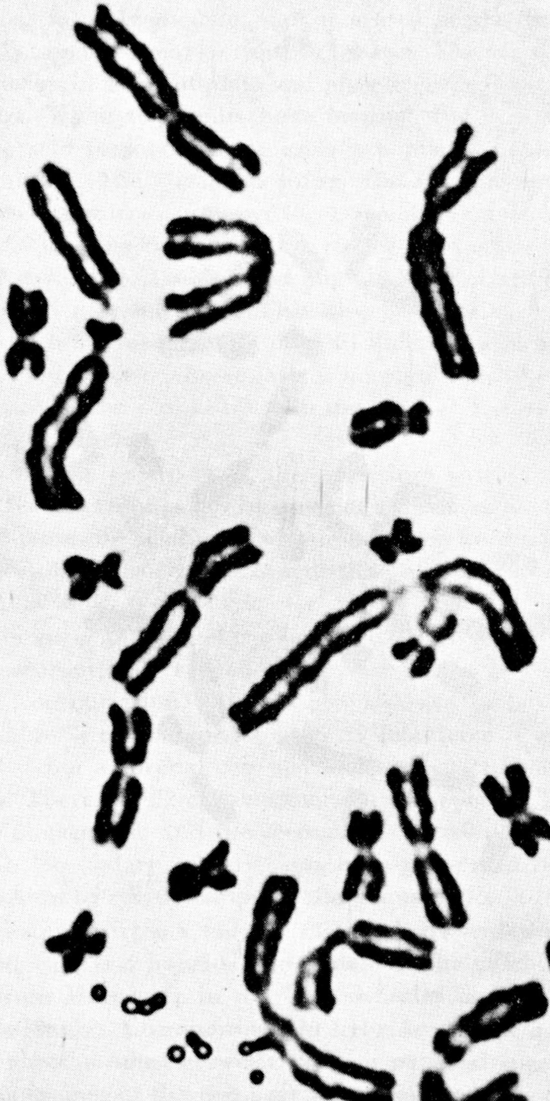

FIGURE 2. *Bufo luetkeni* × *Bufo americanus*.

the hybrid is false. In hybridization experiments performed to determine relationships, it is important to be able to distinguish between true and false hybrids.

False hybrids are encountered from time to time in hybridization experiments. A cell from a triploid hybrid tadpole is presented in Figure 3. This hybrid resulted from crossing a *Bufo viridis* female from Israel with a *Bufo debilis* male from Texas in the United States. Each of the parental species have 22 chromosomes but the hybrid has 33 chromosomes. One of the species contributed two sets of chromosomes. In every case where examination is possible, the female was found to be responsible for contributing the extra set of chromosomes. In *Bufo*, triploids are produced from diploid eggs and haploid sperm. Pentaploid hybrids are produced from tetraploid eggs and haploid sperm. I have never found any evidence for polyspermy in *Bufo*.

A metaphase C configuration of another hybrid tadpole is presented in Figure 4. The female was *Bufo viridis* from Israel and the male was *Bufo mauritanicus* from Morrocco. The hybrid has 55 chromosomes and is a pentaploid. False hybrids are difficult to identify unless the chromosome number is determined. Their external morphology is very similar to true hybrids and they are even capable of metamorphosing.

Chromosomes are easy to obtain and count so the number of chromosomes in a species may readily be ascertained. In order to compare the chromosomes between species, however, a more thorough analysis must be performed. To measure the chromosomes, the negative is placed on an overhead projector and the image is projected on a glass plate with a paper backing. This is a good method to magnify the chromosomes and reduce the error involved in measuring. Chromosomes were measured using a "map measurer" directly on the paper of this rear projection system (Figure 5). It is a good idea to print the negative and number the chromosomes to avoid confusion. In order to reduce the error of measurement, each arm of every chromosome is measured. An average of the two longest arms and the two shortest arms is taken. The length of the chromosome is found by adding the long and short arms. The centromeric ratios are found by dividing the long arms by the short arms. The amount of condensation or contraction of the chromosomes is fairly constant within any one cell but varies between cells even on the same slide, therefore, proportions must be used. I have used two different proportion methods. The first method is to give the longest chromosome a value of 100% and each of the other chromosomes is given as a percentage of the longest chromosome. This method is good for comparing species having a similar number of chromosomes and the size of the longest chromosome is fairly constant between the species. Another method is to add all the measurements from all the

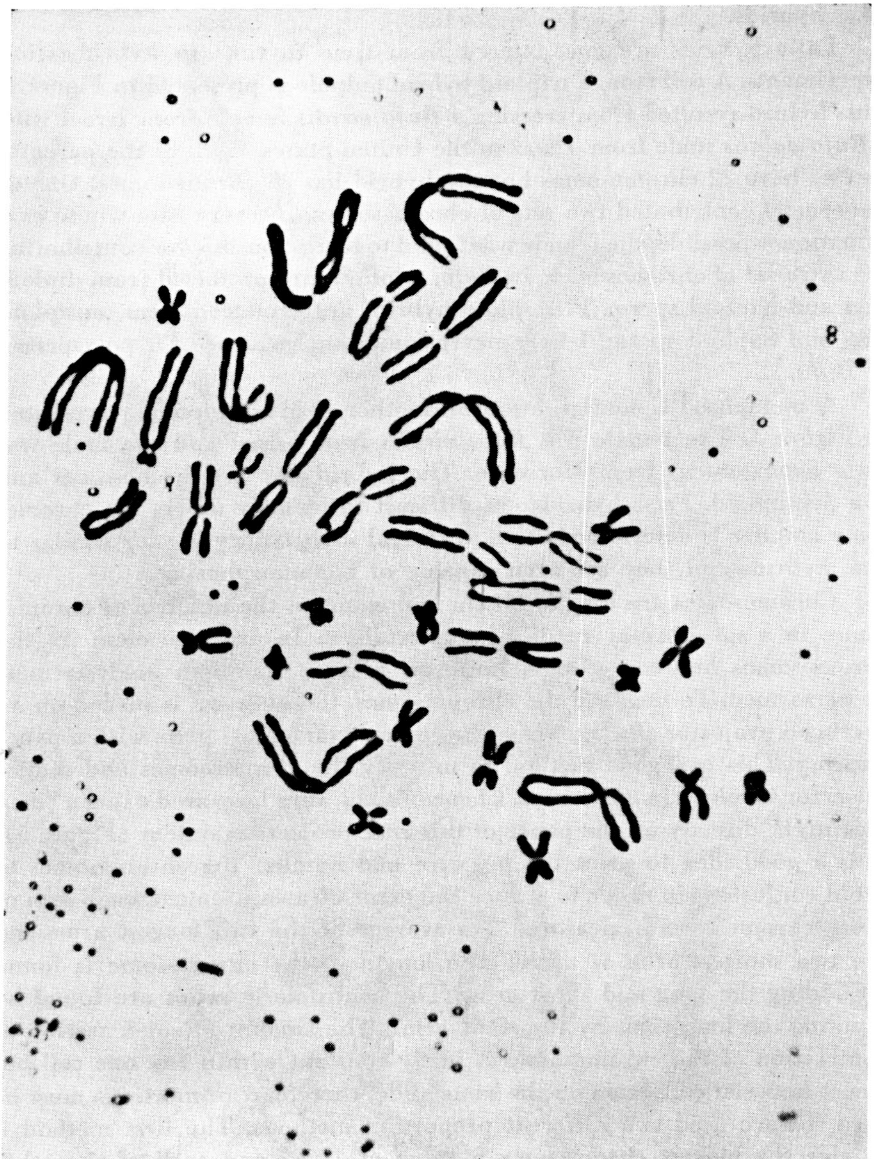

FIGURE 3. *Bufo debilis* × *Bufo viridis.*

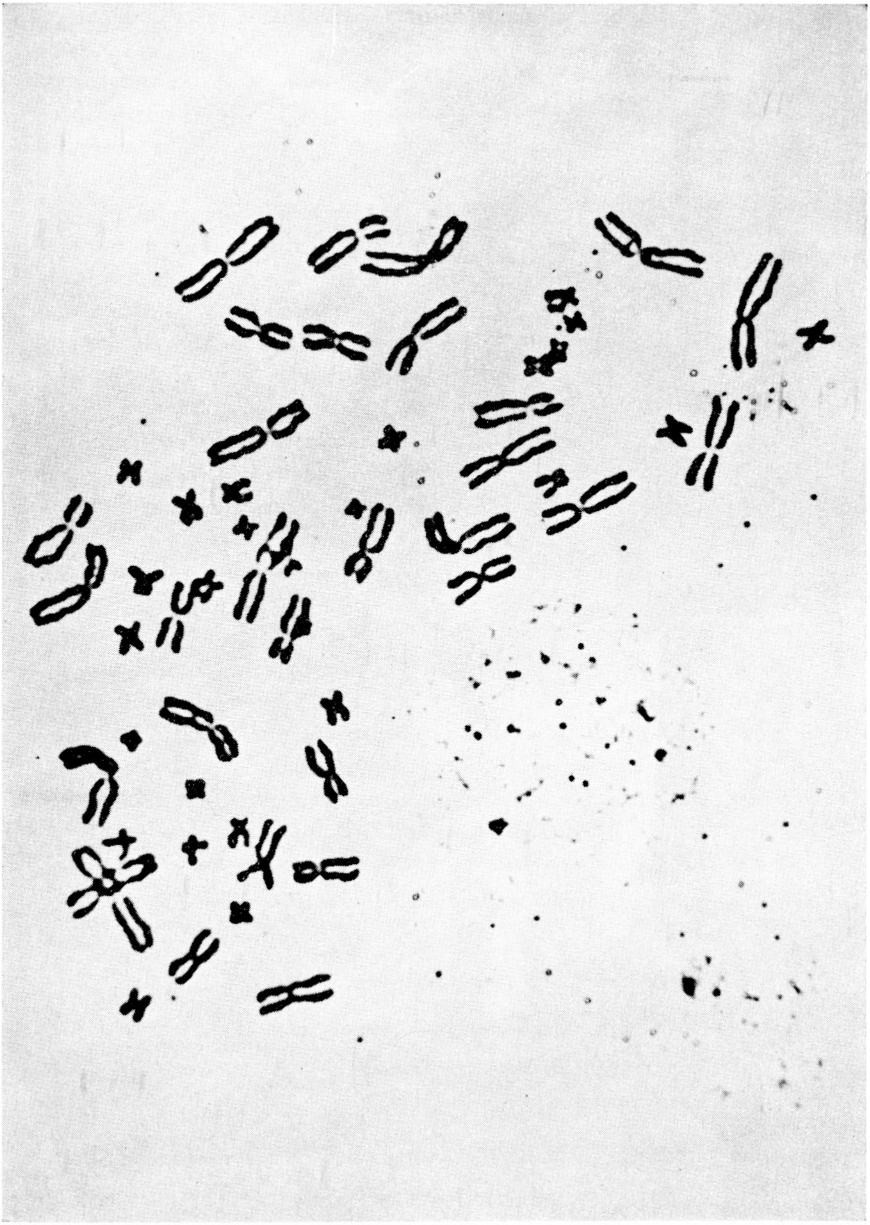

FIGURE 4. *Bufo mauritanicus* × *Bufo viridis.*

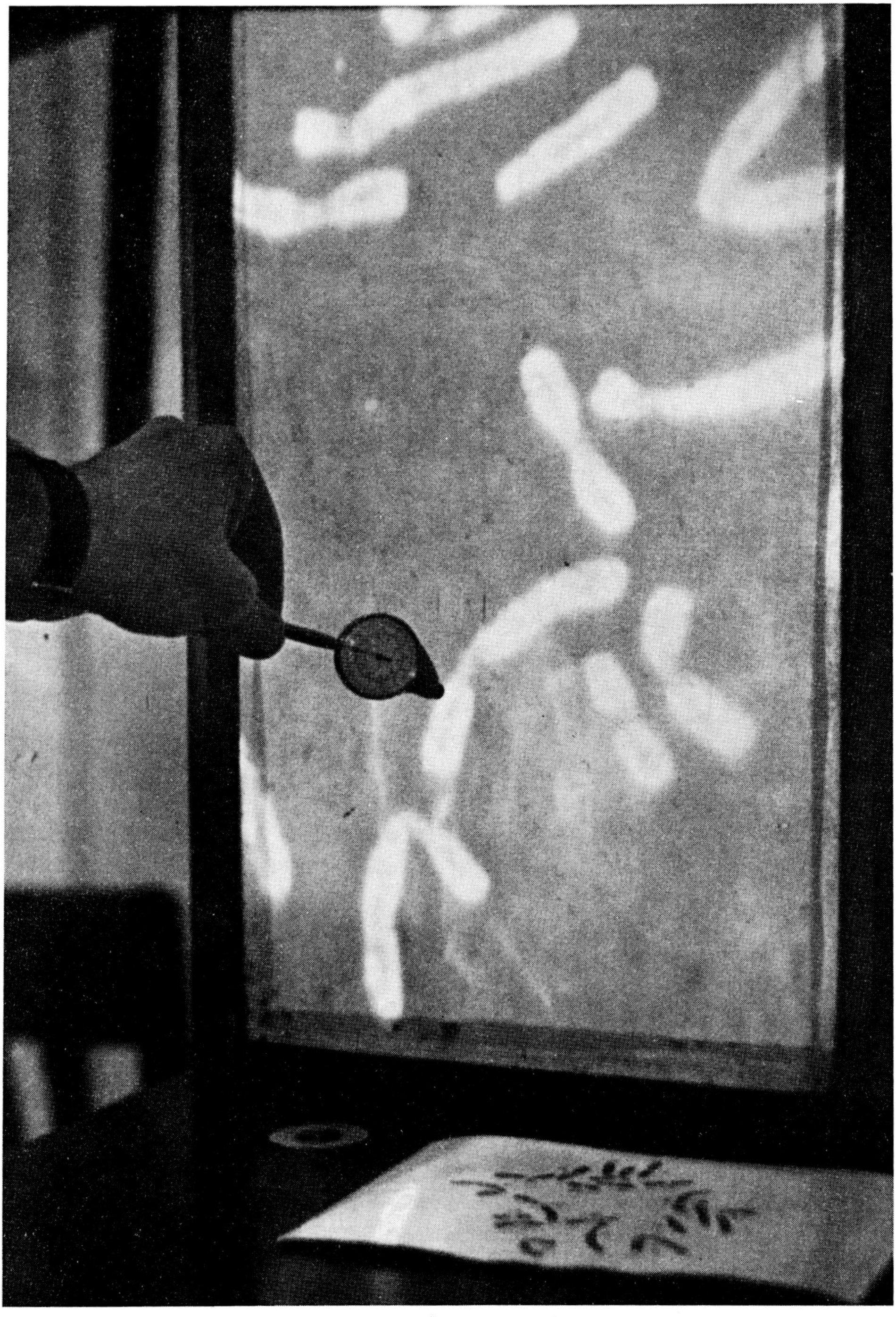

Figure 5. See text (p. 33).

chromosomes together and consider this value as 100%. Each chromosome is expressed as a percentage of the total chromosome length. After the chromosomes have been measured and assigned percentage values and centromeric ratios, the homologous chromosomes are paired according to size, centromeric ratio, and any other evident morphological characters. Since only good, typical cells are analysed, the linear arrangement of the chromosome pairs may be called a karyotype.

A series of karyotypes of some species of *Bufo* is presented in Figure 6. The chromosomes are arranged in homologous pairs in order of size. Except for secondary constrictions, the karyotypes are all quite similar. Karyotypes can, of course, be arranged visually without doing the tedious measuring and calculating. In most cases, visual linear arrangements and calculated arrangements are the same. However, if the calculations are performed, it is very easy to construct idiograms which are essentially graphical interpretations of the karyotypes.

The construction of the idiograms in Figure 7 involved the averaging of the homologous chromosomes and their centromeric ratios. Each bar in the idiogram represents a chromosome pair. The number 6 chromosomes of *B. crucifer* are quite different in the males which I studied. They were not of a similar enough size to be considered homologous. I have some evidence that this non-homologous pair may be related to sex. This is the only species of the sixty *Bufo* species which I have examined that may have a non-homologous pair of chromosomes. The dotted lines (Figure 7) represent percentages of 100 on the top, 50 and 40. Chromosomes 50% or more of the longest chromosome are considered to be large. Intermediate chromosomes have a length between 40 and 50%, and if the length is less than 40% the chromosomes are considered to be small. Five large pairs, one intermediate pair, and five smaller pairs occur in most of the *Bufo* species which I have studied. Secondary constrictions are indicated by voids in the bars. Measurements from the two best karyotypes were averaged for each idiogram to reduce the variation.

Variation between cells or even in the same cell is a problem. Some of the variation may be attributed to error in measurement but there seems to be a certain amount of variation in the chromosomes themselves. In order to determine the amount of variation involved, I analyzed 291 chromosome pairs from 28 triploid hybrid tadpoles. Two chromosomes from each set of three similar chromosomes should have been identical. Any differences should be the result of errors in measurement or natural variation. It was found that the centromeric ratio variation between the identical chromosomes varied from 0 to 1.4. A ratio difference of .5 encompassed 97.6% of the 291 chromosome pairs analysed. The percentage differences varied from 0 to 10. Since these chromosomes were supposed to

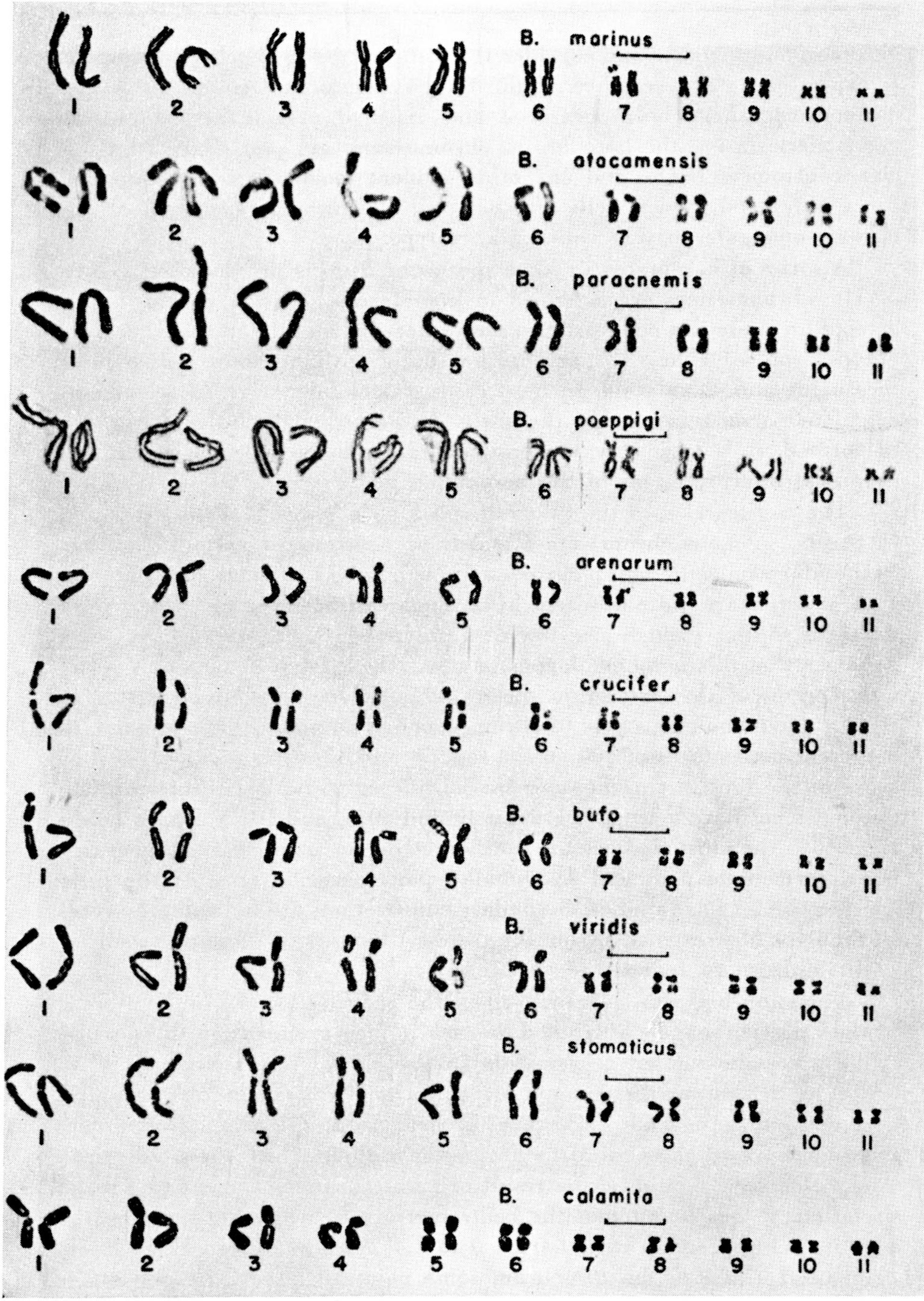
B. marinus
1
2
3
4
5
6
7
8
9
10
11
B. atacamensis
B. paracnemis
B. poeppigi
B. arenarum
B. crucifer
B. bufo
B. viridis
B. stomaticus
B. calamita

FIGURE 6. A series of karyotypes of some species of *Bufo*.

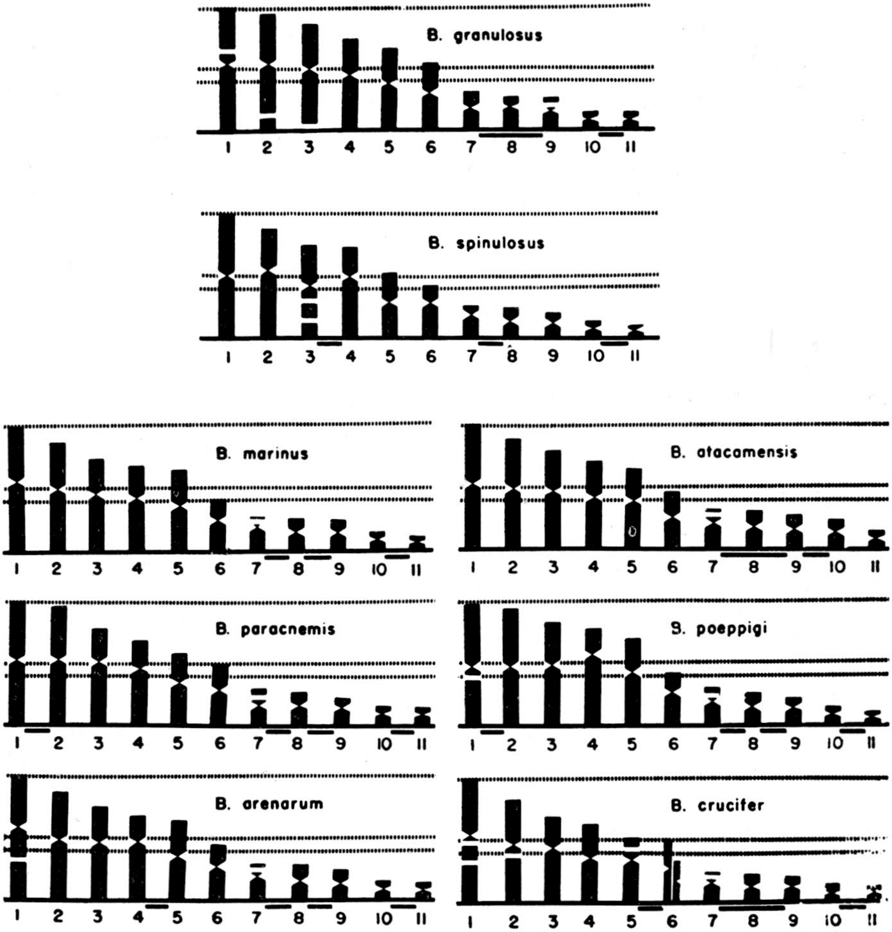
B. granulosus
1 2 3 4 5 6 7 8 9 10 11
B. spinulosus
1 2 3 4 5 6 7 8 9 10 11
B. marinus
1 2 3 4 5 6 7 8 9 10 11
B. atacamensis
1 2 3 4 5 6 7 8 9 10 11
B. paracnemis
1 2 3 4 5 6 7 8 9 10 11
B. poeppigi
1 2 3 4 5 6 7 8 9 10 11
B. arenarum
1 2 3 4 5 6 7 8 9 10 11
B. crucifer
1 2 3 4 5 6 7 8 9 10 11

FIGURE 7. See text (p. 37).

be identical, a ratio variation of .5 or a percentage variation of 10 must be considered to be within the expected variation. When this large variation was considered, it was not possible to use centromeric ratio or percentage length to outline evolution in the genus *Bufo*. This method of analysis is only useful in comparing species having more variable karyotypes.

Secondary constrictions were found in most of the species of *Bufo* which I examined. All the species whose idiograms are presented in Figure 7 have a secondary constriction in chromosome 7. These constrictions are probably homologous and indicate that these species may have been derived from a common ancestor which possessed this constriction. Other secondary constrictions are evident in some of the species and these other secondary constrictions are found in species which do not have a constriction in chromosome 7. In many instances, species with similar secondary constrictions have been found to be closely related by other investigators.

The temporary slides will remain in good condition for about three months. There is a very simple method for making the slides permanent. The slide is submerged in liquid nitrogen until all boiling ceases. The cover glass cement is scraped off with a razor blade and the cover slip is popped off by prying one corner of the cover slip up with a razor blade. The slide is then submerged in absolute alcohol for one minute. A new cover slip is mounted on the slide with a drop of diaphane. If care is taken, there is never any alteration of the chromosomes during this process.

The study of chromosomes is only one aspect in an evolutionary treatment of any taxonomic group. Ideally, entire organisms should be studied in every aspect of their biology. It is only after definitive studies of as many aspects as possible that homologies can be separated from parallelism, convergence, mimicry, or chance. Several sources of evidence should be applied in the formation of any phylogeny.